ISSN: 2287-688X

Annals of
Plant Sciences

Volume 15, Issue 08 (2026) pp. 14-23

Research Article

# New Insights on Pollinia Germination and Cryopreservation of a *Decalepis arayalpathra* - A Critically Endangered Taxon

**Sumalatha, B.S[1], Gokul, S.[2&3] and Rajasekharan, P.E[3]**

[1]*Department of Botany, Bangalore University, Bangalore, India - 560 056.*

[2]*School for Conservation of Natural Resources, Repository for Medicinal Resources, Trans-Disciplinary University (TDU-FRLHT), Bangalore 560 064, India.*

[3]*In-vitro conservation and cryopreservation Unit, Division of Plant Genetic Resources, IIHR, Bangalore 560 089, India*

## Abstract

*Decalepis arayalpathra,* (Apocynaceae s. l.) a critically endangered and narrow endemic taxa confined to Southern Western Ghats, Tamil Nadu and Kerala. The plant has a potential resource for ethno medicinal, phytochemical, economical and biological importance. The present work was assessed on the pollen morphology, cryopreservation (-196°C), pre- and post-*in vitro* germination studies, under different temperature regimes (-20°C and -80°C) and standardization of germination medium to preserve the pollinia. Each single pollinia consist of 12-22 pollen tetrads. The maximum germination of pollen was achieved in PGM2 15% (Brewbaker-Kwack medium) in absence of potassium nitrate. The results obtained by the process of cryopreservation resulted that the fresh pollen attained 52.86% of germination as compared with those pollen stored under temperature regimes -20°C and -80°C, which literally failed to germinate beyond 2nd and 5th day, respectively. However, the germination percentage of cryopreserved pollens was 52% when stored under -196ºC until 30 days of a month. The present investigation offers the possibility of establishing a pollen preservation protocol for breeding and conservation of this narrow endemic species.

**Keywords:** Pollinia, germination studies, Cryopreservation, Conservation.

## Introduction

*Decalepis arayalpathra* (J. Joseph & V.Chandras.) Venter [= *Janakia arayalpathra* J. Joseph & V. Chandras] belongs to the family Apocynaceae. It is a perennial shrub, grows only on rocky cliffs and slopes at an altitude between 600 – 1400m (Joseph and Chandrasekaran, 1978). It has restricted distribution to Southern Western Ghats of Agasthyamala Biosphere Reserve (ABR) confined to Tirunelveli and Kanyakumari districts [Tamil Nadu], Thiruvananthpuram and Kollam districts of Kerala (Ravikumar. *et al*., 2000; Verma and Sarkar, 2014). It has categorized as Critically Endangered (Molur and Walker, 1996) and declared as "species of high conservation concern" by National Biodiversity Authority of India (Notification 2009, 2011). The recent investigation, states that the species is 'threatened' due to its specific ecological niche and its existence on rocky outcrops, fragmented population and narrow distribution along with root rot disease (Premalatha. *et al*., 2015). The other reason of decline is due to overexploitation of root (Malleshappa, 2013; Sarkar, 2012) where the local Kani tribes inhabiting in ABR, habits tubers for treating peptic ulcer, cancer-like afflictions and as a rejuvenating tonic (Pushpangadan. *et al*., 1990). However, it has low genetic diversity based on the studies of ISSR and RAPD molecular analysis (Premalatha. *et al*., 2015).

Among the reproductive events in the species, pollination, seed dispersal and seedling

***Corresponding Author:**
**Sumalatha, B.S;**



DOI: 10.5281/zenodo.22124233

establishment are the most serious aspects to be considered. If in case failure of any one of them could lead the species to threatened. Since, there is less studies on pollination biology of wild Plant Genetic Resource (PGR) (Shivanna, 2012). The studies on pollination biology is need of the hour for multiple scientific research areas like systematic botany, paleobotany, paleoecology and pollen analysis for successful conservation and management of any endangered species (Mazari. *et al*., 2017). Hence, pollen cryopreservation is a promising application and cost-effective technique for maintaining genetic diverse stocks of pollens collected from wild for future conservation and a strategy that can also accomplish conservation of Nuclear Genetic Diversity (NGD) to conserve genetic variation expressed through pollen (Rajasekharan, 2015). Application of cryogenic techniques for conservation of wild tropical plant species would enable extended use of the male gametophyte for providing access to the conserved nuclear genetic variability, there by facilitating hybrids genera and genetic enhancement of derived crops, which requires introgression of pollen transmitted genes from wild source (Normah. *et al*., 2013). In other aspects, for long term *ex situ* conservation through cryopreservation eliminates the need for frequent sampling of pollen from the wild for assisted pollination needs, eventually increase the sustainable use of tropical plant resources available *in situ*, and allows normal evolutionary processes to the operated (Engelmann, 2004).

The present investigation is based on the studies of pollen structure; cryopreservation protocols have been developed for pollen. Furthermore, the evaluation of *in vitro* germination of fresh and cryopreserved pollen for various durations will be carried.

## Materials And Methods

The study area is confined to southern tip of Western Ghats, in and around Agasthyamalai Biosphere Reserve that spreads over in two districts of Tamil nadu i.e., Tirunelveli, Kalakad-Mundanthurai Tiger Reserve (KMTR) and Kanyakumari, Kanyakumari Wildlife Sanctuary (KWS). *D. arayalpathra* was collected and conserved in Ethno Medicinal Garden (EMG) field gene bank (Fig. 1a-c) of Trans-Disciplinary University (TDU-FRLHT), Bangalore for further studies.

**Pollinia Collection and Germination:**

To obtain pure samples of pollinia, the flowers were bagged with paper bags at the budding stage. The pollinia, collected from the freshly opened flowers that 1–2 days of after anthesis and brought to the laboratory in sterile petri plates. Petals and translators were carefully removed with sterile needle and were placed on the watch glass. Initial care was being taken to avoid the damage, while excising the pollinia from translocator (Fig. 1d-g). further, the fresh pollinia were mounted on slide to study the morphological characters using Nikon microscope, SMZ 1000 (Japan).

**Pollen Germination Medium (PGM)**

The Brewbaker-Kwack medium was prepared in two ways. 1. PGM1: containing (sucrose 5-30%, boric acid 100 mg/L, calcium nitrate 300 mg/L, potassium nitrate 100 mg/L) and 2. PGM2 containing (sucrose 5-30%, boric acid 100 mg/L, calcium nitrate 300 mg/L) were used for further studies.

**Alexander Differential Staining to Assess Pollen Fertility** (Alexander, 1980)

The stain was prepared by mixing the following constituents: 1. Ethanol (96%)-20 ml 2. Distilled water-50 ml 3. Glycerol-40 ml 4. Acid Fuchsin-100 mg 5. Phenol-5 g 6. Lactic acid-2 ml.

Pollinia is dusted with 1-2 drops of stain on slides and warmed lightly before placing the cover slip. The cover slip was sealed with fixogum to prevent evaporation. Slides were examined after 15-30 minutes. Incubation at $50^{o}$ C for 2-4 h, makes the stain deepens.

***In vitro* Germination and Viability of Pollen:** (Shivanna and Tandon, 2014)

The present investigation was carried by taking the fresh pollinia and were dehydrated by simple air drying for 10 min at 27 ± 2°C by placing them in laminar air flow cabinet. Later, the pollinia were incubated in the presence of PGM1 and PGM2 medium, in addition with

various concentrations of sucrose (5-30%) by hanging drop method. The slides were maintained in a humid chamber at 25 ± 2°C. Further, germinated pollinia was stained with Alexander's stain to check the viability.

**Temperature Regimes for Pollinia Preservation:** (Ganeshan. *et al*., 2008)
About 10-15 dehydrated pollinia were transferred to the cellophane sheet (4×3 cm) and carefully placed in empty gelatin capsules, and sealed with airtight laminated poly aluminum pouches. Further, labelled and were stored, with sufficient number of pouches under different temperature (-20°C, -80°C and -196°C). Initially, care was taken when pouches were placed in the Liquid Nitrogen (LN) canister (Mach SM-43, MVE USA) by gradually plunging inside. Samples were placed in LN for extended periods, to analyze their viability even after prolonged storage. At the time of testing, the pollens were taken out of the cryobiological system and rewarmed to ambient temperature.

**Preparation of Pollinia Sample for Scanning Electron Microscopy:**
The dissected pollinia were rinsed in ethanol (70%): lactic acid in a ratio of 2:1 to remove debris. Further, it was transferred to the cavity slide containing glutaraldehyde 2-5% and were washed with 0.1M phosphate buffer (pH 7.0), there after with 1% Osmium teroxide ($OsO_4$) in the same buffer (pH 7.0) for 30min. The treated samples were rinsed with distilled water for 5 min, followed by dehydrating through an increase gradient of ethanol series concentration (Kuo, 2008). Later, the samples were dried at critical point dryer using $CO_2$ as the transition fluid and then mounted on the stubs with tacky tapes. Specimen was coated with gold using Ion sputterer to a thickness of 30-40nm (Sputter coating Quotum SC 7620). The coated samples were examined with a VEGA3 TESCAN SEM (USA) set between 1.5 to 5 kV acceleration voltage. The angle of tilt varied depending upon the nature of the sample examined.

**Experimental Design and Data Analysis**
The experiment was subjected to analysis of variance (Panse and Sukhatme, 1995) and means were compared by Duncun's Multiple Range test using SAS 9.3 version.

## Results

**Flower and Pollinium Morphology Along with SEM Analysis**
The flowers of *D. arayalpathra* are small in size (3-4 X3-3.5 mm) and cream-yellow in colour. It has a distinct spathulate 'translator' which contains a pair of massulae (Fig. 1d) and together called as pollinium (Fig. 1b). Each flower consists of five pollinium. In each individual pollinium contains free pollen tetrads. These tetrads are grouped together forming pollen mass (Verhoeven and Venter, 1997,1998). Pollinium massulae size varied between 200 - 450 X 60 - 250 µm. A single pollinium massulae contains a row of 12-22 pollen tetrads. The pollen tetrads are tetrahedral in planer view having a smooth surface (Fig. 1e-g). The pores were observed in proximal walls of adjacent tetrads opposite to each other (Fig. 1e). Tetrad were 52.3 ± 5 in length and 72.5 ± 5 µm in breadth.

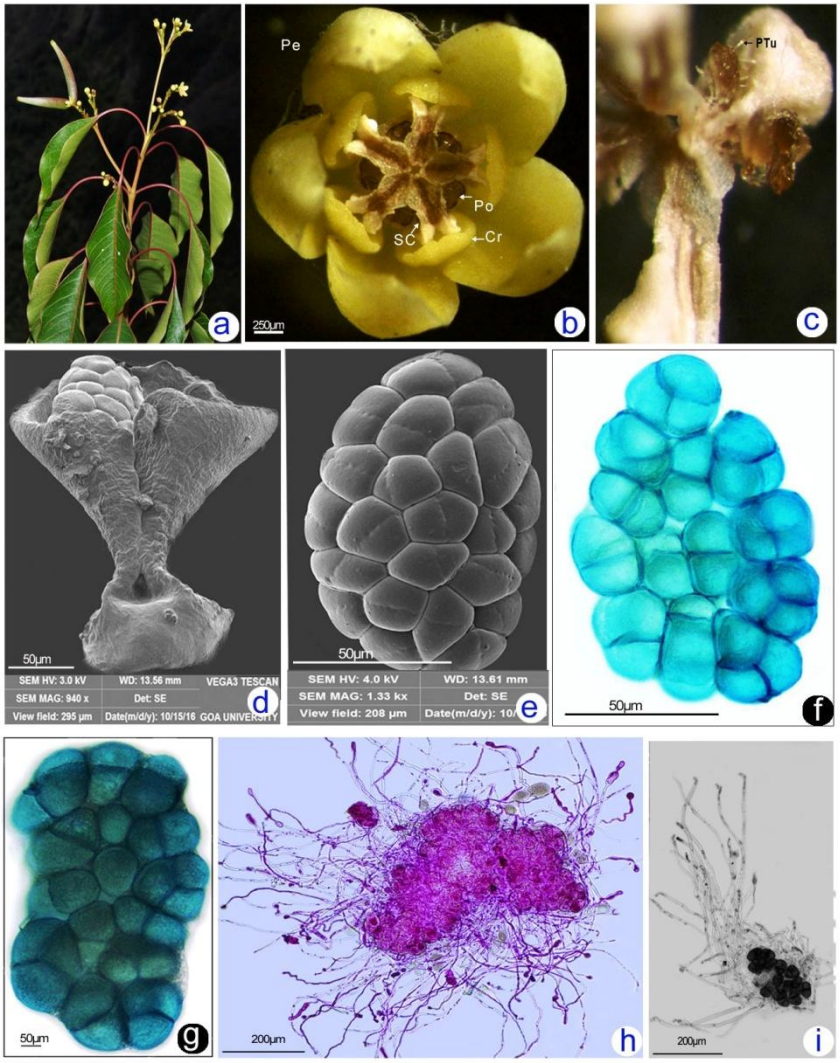


**Figure 1a:** Flowering and fruiting twig of *Decalepis arayalpathra*; **b:** Close-up of flower showing Corona (Cr), Petals (Pe), Pollinium (Po) and Stigmatic Surface (SC); **c:** *In-vivo* pollinium germination on stigmatic surface (PTu-pollen tube); **d**: Pollinium SEM of translator with massulae; **e:** SEM of pollinium; **f-g:** Pollinium stained with Alexander stain showing pollen tetrads mass clear; **h-i:** *In-vitro* pollen germination.

**Effect of Pollen Germination Medium (PGM) on Pollen Germination**

The germination of pollens was recorded with the sucrose supplemented medium ranging from 5-20%. In pollinia, the germination was observed in between 4 to 20 hour of incubation period. The sucrose concentration of 10% showed germination with PGM2, where as in PGM1 failed to germinate. The PGM2 medium supplemented with 15% sucrose reinforced maximum germination and was optimal medium for *in vitro* germination of the pollen (Fig. 2).

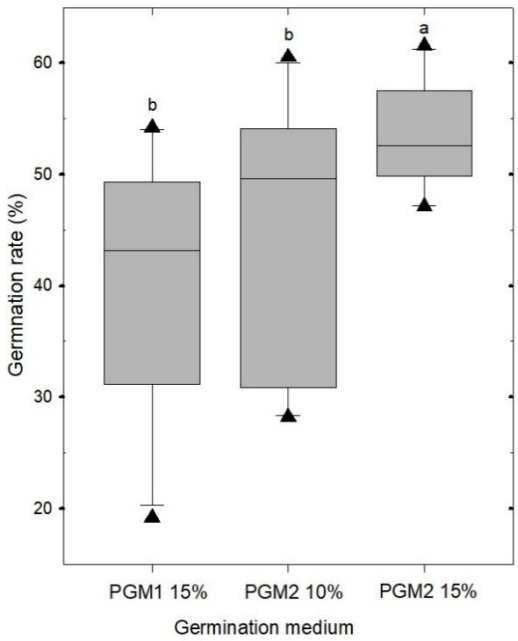


**Figure 2:** Effect of germination medium and sucrose concentration on *in vitro* pollen germination in *D. arayalpathra*. The bar represents the range of germination (n=10) after 24 days, while the horizontal line inside the bar represents, the mean value and also the error bar denotes standard error. Different letters denote significant differences based on Duncan's multiple range test, $p = 0.05$

The highest germination of pollen tube was recorded in PGM2 with 15% and with maximum length of pollen tube 410 - 415 µm (Fig. 1h & i) around at 18 h (Fig. 3). The least germination and pollen tube length was observed in PGM1, whereas in PGM2 at 15% and 10% respectively.

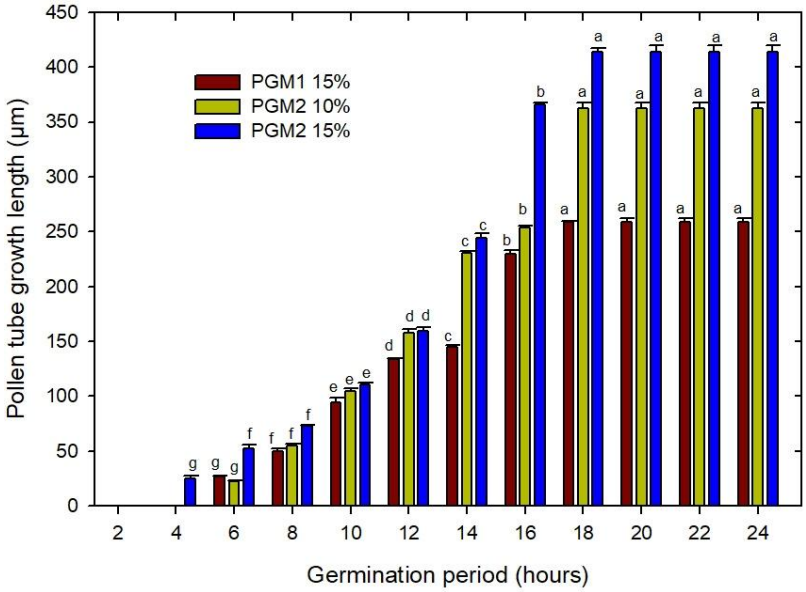


**Figure 3:** Effect of germination medium on pollen length of *D. arayalpathra* at different sucrose concentrations, when incubated up to 24 hours. The error bar denotes the standard error (n=3), different letters denote significant differences compared within different PGM experiments based on Duncan's multiple range test, $p = 0.05$.

**Pollen Germination Viability After Storage**

The pollens were stained with Alexander's stain to check the viability, retained pink in colour. Non-viable pollen stained green or

sometimes remained colourless (Tejavathi et.al., 2019). The germination percentage of fresh *D. arayalpathra* pollen was 52.86%. In the case of stored pollens, the viability of the pollen gradually decreased under the temperature at −20°C and −80°C (Fig. 4). The significant negative correlation was observed between germination of pollen grains and storage temperature in a period of twenty days' storage (Fig 4).

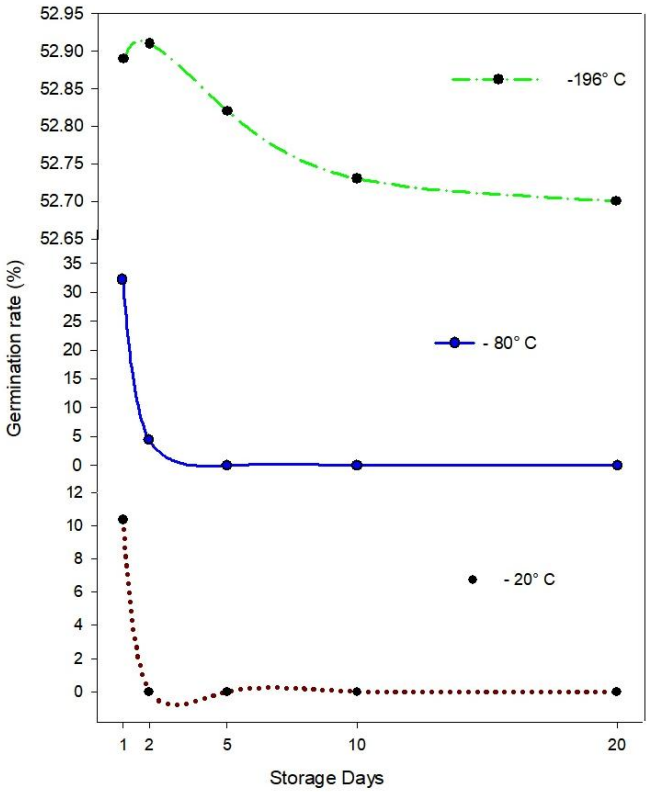


**Figure 4:** The germination percentage of *D. arayalpathra* under different conditions and times.

However, among all the three storage temperatures such as -20°C (-0.47513), -80°C (-0.54520), and -196°C (-0.9222), the germination of pollinia declined after 20 days of storage in natural condition. Significantly, the cryopreserved pollinia had shown germination even after 20 days of preservation and lasted for 3 months (Fig. 5) with the significant correlation at 0.78** with the storage months.

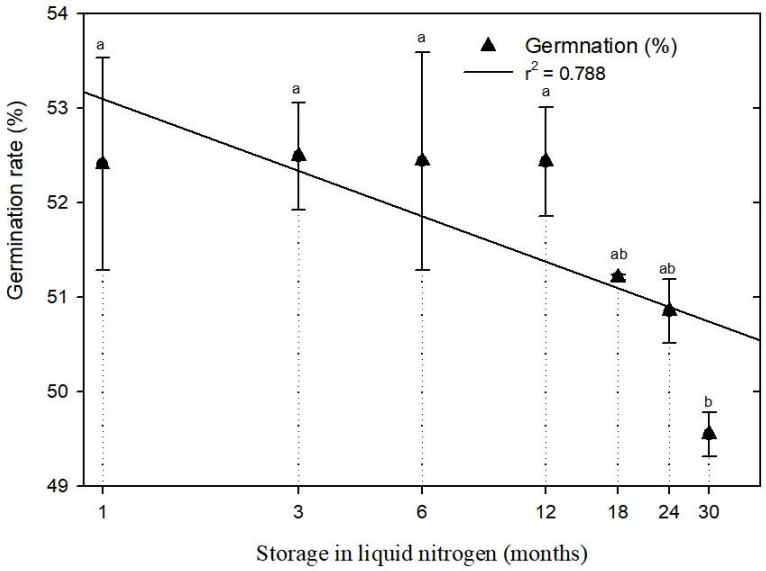


**Figure 5:** Effect of Liquid Nitrogen (LN) on the pollen germination rate of *D. arayalpathra*. The error bar denotes the standard error (n=3), different letters denote significant differences based on Duncan's multiple range test, $p$ = 0.05.

## Discussion

The pollens of tropical RET species are amenable to cryopreservation and impact of such storage over long period of duration needs to be investigated. Such studies are ideal and essential for establishing pollen cryo banks for RET species. It also helps for assisted pollinations and conserving the nuclear genetic variability, by maintaining genetically diverse stocks of pollen collected from different populations in the region specific to *ex-situ* conservation programs.

The pollinium wall present in the Asclepiadoideae referred as a pollinial pellicle (Swarupanandan. *et al*., 1996), an envelope found to be sporopollenin in nature, which accounts for preservation and durability during pollen processing and are highly resistant to decay especially under anaerobic conditions due to enzymatic degradation by fungi and other microorganism (Shukla. *et al*., 1998). The exine layer of the pollen (Fig.1 e-g) consists of a solid stratum (tectum) subtended by a granular stratum (Verhoeven and Venter, 1998). The thin inner proximal layers of pollen are considered as endexine (Dannenbaum and Schill, 1991). The SEM image also reveals the translator bearing massula and individual focused pollinia (Fig. 1. d, e).

The results obtained derives that during hot climate, the nectary content evaporates and become highly concentrated sugar solution, which thereby inhibits pollen germination (Southwick, 1983). In *Asclepias syrica,* observed that the pollen germination was maximum in 15% of sucrose and while, sucrose concentrations below 5% did not support germination, and above 30%, germination was slow (Kevan. *et al*., 1989). Similarly, in *D. arayalpathra* the media PGM2 supplemented with 15% sucrose had shown maximum pollen germination (Fig. 2 and 3). Whereas, PGM1 showed minimal growth. Similar type of result was tabulated in *Moringa oleifera* where Potassium nitrate did not show any effect in pollen germination (Bhattacharya and Mandal, 2004). Generally, sucrose is considered to have two different functions in the medium, as an energy source and osmoticum. The sucrose supplemented externally helps in maintains the osmotic pressure and also provides the essential substrates for metabolism of pollen (Shivanna and Tandon, 2014). The pollen tetrads germinated in mass (Fig.1 h-i), which was in accordance with the results observed in *Periploca graceae* (Dane, 2000) and Asclepiadaceous pollens (Ganeshan. *et al*., 2008). In other words, several factors affect the *in vitro* germination of pollen grain viz genotype, temperature and time of

conservation (Benson, 2008; Patel and Mankad, 2014; Popova. *et al*., 2016; Xu. *et al*., 2014) (Fig. 4 and 5). The pollen germination profile of fresh and cryopreserved pollen was at par. *In vitro* germination of fresh pollen grain was higher (52.90%) and marginally decreased to 52.70% after the 10 days of cryopreserved. This difference could be attributed due to the damages exerted by freezing and storage which may result in the formation of ice crystals and eventually ruptures the tissues and its structure.

The present investigation offers the possibility of applying efficient pollen cryopreservation protocols for breeding and for conservation of the endemic, endangered forest species. This technique conserves variability in the male gametophyte and helps to conserve the plants in the form of germplasm.

## Acknowledgement

We profusely thank the Tamil Nadu State Biodiversity Authority (TNBB52/2011) and Principal Chief Conservator of Forests (WL5/63687/2009), Tamil Nadu Forest Department, for permitting us to carry out this research work. This study is a part of the research work of the project funded by the Department of Science and Technology (SR/SO/PS-25/08), Government of India, New Delhi.

## Reference

1. Alexander, M.P. "A versatile stain for pollen, fungi, yeast and bacteria." *Stain Technol*, 55(1980): 13–18.
2. Benson, E.E. "Cryopreservation Theory, in: Reed, B.M. (Ed.), Plant Cryopreservation: A Practical Guide." *Springer New York, New York*, (2008):15–32.
3. Bhattacharya, A., Mandal, S. "Pollination, pollen germination and stigma receptivity in *Moringa oleifera* Lamk." *Grana,*43 (2004): 48–56.
4. Chacko, S., Sethuraman, M.G. and George, V. "Phytochemical investigation of the roots of Janakia arayalpathra Joseph & Chandrasekaran." *Herba Pol.*. 46(2000):212–219.
5. Dane, F. "*In Situ* Germination of Pollen Tetrads in Periploca graceae L. (Periplocaceae)." *Turkish J. Biol.*24 (2000):337–343.
6. Dannenbaum, C., Schill, R. "The development of pollen tetrads and pollinia in Asclepiadaceae." *Bibl. Bot.*34(1991):1–138.
7. Engelmann, F. "Plant Plant cryopreservation: Progress and prospects." *Vitr. Cell. Dev. Biol.* 40(2004):427–433.
8. Ganeshan, S., Rajasekharan, P.E., Shashikumar, S. and Decruze, W. "Cryopreservation of Pollen, in: Reed, B.M. (Ed.), Plant Cryopreservation: A Practical Guide," *Springer New York, New York*, (2008):443–464.
9. Joseph, J., Chandrasekaran, V. "Janakia arayalpathra - A New Genus and Species of Periplocaceae from Kerala, South India." *J. Indian Bot. Soc.* 57(1978):308–312.
10. Kevan, P.G., Eisikowitet, D. and Rathwella, B. "The role of nectar in the germination of pollen in *Asclepias syrica* L." *Bot. Gaz,* 150 (1989):266–270.
11. Kuo, J. "Electron Microscopy." 2nd Edi. ed. *Humana Press, Totowa, New Jersey*. (2008).
12. Malleshappa, H. "Distribution, population and ecology of *Decalepis aryalpathra* (Joseph & Chandras.) Vent. (Periplocoideae) in Kalakad Mundanthurai Tiger Reserve, southern Western Ghats, India." *Indian Forestry*. 39(2013): 483–486.
13. Mazari, P., Liu, Q., Khan, M.A., Sadia, S. and Ahmad, L. "Pollen Morphology and Pollen Fertility Estimation of Three Medicinal Plant Species of Hypericum L. from Kaghan Valley, Northern Pakistan." *Am. J. Plant Sci.*08, (2017):3073–3083.
14. Molur, S., Walker, S. "Conservation Assessment and Management Plan (CAMP II) for Selected Species of Medicinal Plants of Southern India. "Bangalore. (1996).
15. Normah, M.N., Chin, H.F. and Reed, B.M. "Conservation of Tropical Plant Species." *Springer New York, New York*, (2013).
16. Notification "Kerala Biodiversity Notification. Ministry of Environment and Forests." *Gaz. India,* New Delhi , (2009):1–4.
17. Notification,"Tamil Nadu Biodiversity Notification. Ministry of Environment and Forests." *Gaz. India,* New Delhi (2011):1–3.
18. Panse, V.G., Sukhatme, P. V, "Statistical

methods for agricultural workers." *Indian Council of Agricultural Research, New Delhi* (1995).
19. Patel, R.G., Mankad, A.U. "*In Vitro* Pollen Germination - A Review." *Int. J. Sci. Res.* 3(2014): 2012–2015.
20. Popova, E., Kim, H.H., Saxena, P.K., Engelmann, F. and Pritchard, H.W. "Frozen beauty: The cryobiotechnology of orchid diversity." *Biotechnol. Adv.* 34 (2016):380–403.
21. Premalatha, K., Gokul, S., Kumar, A., Mishra, P.P., Mishra, P.P., Ravikumar, K. and Kalra, A. "Molecular profiling of fungal assemblages in the healthy and infected roots of Decalepis arayalpathra (J. Joseph & V. Chandras.) Venter, an endemic and endangered ethnomedicinal plant from Western Ghats",. *India. Ann. Microbiol.* 65(2015):785–797.
22. Pushpangadan, P., Rajasekharan, A., Ratheeshkumar, P.K., Jawahar, C.R., Radhakrishnan, K., Nair, C.P., Amma, L.S. and Bhatt, A. V, "Amrithapala" (Janakia arayalpathra, Joseph & Chandrasekharan), a new drug from the Kani tribe of Kerala." *Anc. Sci. Life.* 9(1990): 212–214.
23. Rajasekharan, P.E. "Gene Banking for Ex Situ Conservation of Plant Genetic Resources", in: Bahadur, B., Rajam, M.V., Sahijram, L., Krishnamurthy, K.V. (Eds.), Plant Biology and Biotechnology. Springer, New Delhi, Page. (2015):445–449.
24. Ravikumar, K., Ved, D.K., Vijayasankar, R. and Udayan, P.S. "Illustrated field guide to 100 Red listed medicinal plants of Conservation Concern in southern India." *Foundation for Revitalisation of Local Health Tradition,* Bangalore., (2000).
25. Sarkar, M.K. "Biodiversity Governance for Managing Endemic and Threatened Medicinal Plants in India – A Geoinformatic Approach", 2nd ed. *National Biodiversity Authority*, India, Chennai. (2012).
26. Shine, V.J.J., Shyamal, S., Latha, P.G. and Rajasekharan, S. "Gastric Antisecretory and Antiulcer Activities of *Decalepis arayalpathra*." *Pharm. Biol.* 45(2007):210–216.
27. Shivanna, K.R. "Reproductive Ecology and Conservation of Plant Genetic Resources of the Wild." *Indian Soc. Plant Genet. Resour.* 25(2012):75–84.
28. Shivanna, K.R., Tandon, R., Reproductive Ecology of Flowering Plants: A Manual. (2014).
29. Shukla, A.K., Vijayaraghavan, M.R. and Chaudhry, B. "Biology of Pollen." APH Publishing. (1998).
30. Southwick, E.E. "Nectar biology and nectar feeders of common milkweed, *Asclepias syriaca* L." *Bull. Torrey Bot. Club,*110(1983):324–334.
31. Swarupanandan, K., Mangaly, J.K., Sonny, T.K., Kishorekumar, K. and Basha, S.C. "The subfamilial and tribal classification of the family Asclepiadaceae." *Bot. J. Linn. Soc.*120 (1996):327–69.
32. Tejavathi D.H., Sumalatha B.S, Nijagunaiah R., Anitha P. and Gayatramma K.. "Studies on the factors that restrict sexual reproducito and fruit setting in *Memecylon flavescens*: an endangered taxon." *Journal of Tropical Forestry and Environment*. 9.2(2019).: 101-107.
33. Verhoeven, R.L., Venter, H.J.T. "The translator in Raphionacme (Periplocaceae)." *South African J. Bot.* 63(1997):46–54.
34. Verhoeven, R.L., Venter, H.J.T., Pollinium structure in Periplocoideae (Apocynaceae). *Grana,*37(1998):1–14.
35. Verma, R.S., Mishra, P., Kumar, A., Chauhan, A., Padalia, R.C. and Sundaresan, V. "Chemical composition of root aroma of *Decalepis arayalpathra* (J. Joseph and V. Chandras.) Venter, an endemic and endangered ethnomedicinal plant from Western Ghats," *India. Nat. Prod. Res.* 29. 5(2014):394-395.
36. Verma, S.K., Sarkar, M.K. "*Decalepis arayalpathra* (J. Joseph & V. Chandras.) Venter, an endemic and endangered ethno medicinal plant from Western Ghats,." *India. Nat. Prod. Res.* (2014):1–2.
37. Xu, J., Li, B., Liu, Q., Shi, Y., Peng, J., Jia, M. and Liu, Y. "Wide-scale pollen banking of ornamental plants through cryopreservation." *Cryo. Lett.* 35(2014):312–319.

**Source of support:** Nil;
**Conflict of interest:** The authors declare no conflict of interests.

**Cite this article as:**
Sumalatha, B.S., S. Gokul. and Rajasekharan, P.E. "New Insights on Pollinia Germination and Cryopreservation of a *Decalepis arayalpathra* - A Critically Endangered Taxon." *Annals of Plant Sciences.*15.08 (2026): pp. 14-23.